PHOTOLYSIS AT VERY LOW TEMPERATURES OF CO-LIGANDED CYTOCHROME OXIDASE (CYTOCHROME *d*) IN OXYGEN-LIMITED *ESCHERICHIA COLI*

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1. Introduction

Cytochrome *c* oxidase (EC 1.9.3.1) is the terminal enzyme of the respiratory chain of mitochondria [1] and certain bacteria [2]. The extensively studied mammalian enzyme contains 2 haems (a,a_3) and 2 mol copper. In the reaction with O_2, the reactive centre consists of the coupled metal ion pair, cytochrome a_3 and the 'EPR invisible' Cu_{a_3}. It is to the former that CO also binds in a light-reversible fashion [3]. The photodissociation of CO–cytochrome oxidase at low temperature was first attempted in [4], where the dissociated complex formed by flash photolysis did not recombine below 150 K. Much lower temperatures [5] afforded a clear distinction between cytochrome aa_3 (where recombination occurred only as the temperature was raised from 150–200 K) and a wide range of free and protein-bound ferrous haems (including myoglobin, haemoglobin and protohaem), in which CO recombination occurred with a mid-point of 25–30 K [6]. These differences in kinetic behaviour allow cytochromes a and a_3 to be quantified in the presence of blood pigments [7].

Under anaerobic [8] or O_2-limited conditions [9,10], *Escherichia coli* forms (in addition to cytochrome oxidase o) an alternative oxidase with a higher O_2 affinity [10], namely cytochrome d. In [9,11,12], we were unable to detect photolysis of the CO-liganded cytochrome d at ~143 K. One interpretation of this result is that the intensities of light used were insufficient: the insensitivity to photolysis of the CO complexes of cytochrome d [13] and other haem proteins [14] has been reported. However, photolysis at this temperature did give rise to a spectrally distinct form of the oxidase in the presence of O_2 [9,11], suggesting photolysis of the CO compound and ligand exchange.

Here, we show that much lower temperatures are required to observe the photolysis and subsequent recombination of CO with cytochrome d, making it unique in this respect among oxidases studied to date, and relating it to the low temperature properties of myoglobin and haemoglobin to a greater extent than to cytochrome aa_3.

2. Experimental

2.1. *Growth and harvesting of bacteria*

Escherichia coli K12 (strain A1002) was maintained and grown in the medium of [12], but with sodium succinate was lowered to 20 mM. Batch cultures (6 l) were grown in a New Brunswick fermenter; sparging with sterile air was at 0.6 l air/min and the stirring speed was sufficient only to prevent sedimentation of cells. The O_2 transfer rate thus obtained was typically 8.4 mmol . l^{-1} . h^{-1}; growth under these conditions is O_2-limited [9]. Cells were harvested 19–23 h after inoculation with a stationary phase starter culture, when E_{600} (undiluted; 1 cm cuvettes) was 0.4–0.8. Harvesting by centrifugation, washing and resuspension of cells in buffer containing 30% (v/v) ethylene glycol as cryosolvent was as in [12], except at 20 g (wet wt) cells/100 ml suspension.

2.2. *Preparation of cells for low temperature experiments*

A cell suspension (8 ml) was further concentrated

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by centrifuging and suspending the cells in 1 ml supernatant, giving a total cell protein concentration of ~ 40 mg/ml. A portion (1 ml) was reduced by respiration in the presence of 20 mM sodium succinate for 30 min in the barrel of a hypodermic syringe. After sparging with a slow stream of CO for 2.5–5 min (giving ~1.2 mM CO) the sample was injected from the syringe into a small copper sample holder (1 mm pathlength; 0.05 ml) fitted with 'Cellotape' windows and frozen on dry ice in the dark. In experiments where photolysis was performed in the presence of O_2, the CO-saturated sample was equilibrated at −25°C for 5 min before stirring in O_2 for 30 s (thereby introducing the ligand without displacing the bound CO). The sample was then injected into a precooled sample holder (cuvette) and frozen on dry ice.

2.3. *Biophysical techniques*

Reflectance spectra were recorded with a Johnson Foundation microprocessor-controlled split-beam spectrophotometer. The optical configuration (fig.1) allowed reflectance spectroscopy from the front face and photolysis on the back face of the sample, using a liquid-dye laser that provided about 50 mJ at 580 nm. The reference cuvette contained 50% (v/v) buttermilk. Both cuvettes were mounted in a sample holder at the base of an Air Products LT-3-110 Helitran, providing temperatures down to 4 K [15].

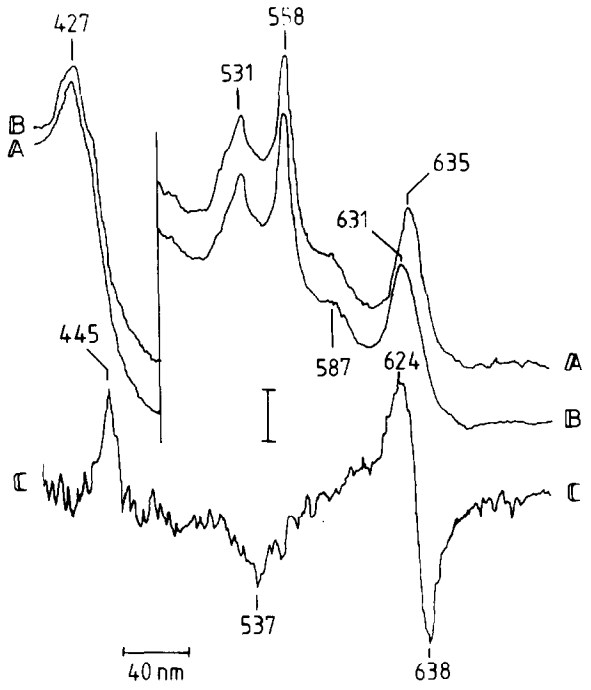

Fig.2. Reflectance spectra of oxygen-limited *E. coli* and the effect of flash photolysis: (A) absolute spectrum of CO-liganded, succinate-reduced cells at 4 K; (B) same sample after one flash of the liquid dye laser; (C) the difference spectrum (photolysed minus CO-reduced). The vertical bar denotes 0.01 ΔA (α and β regions or 0.02 (Soret)) in (A) and (B) and 0.004 ΔA in (C). Scan rate was 2.4 nm/s; protein was 43 mg/ml.

3. Results

Fig.2A shows the absolute spectrum at 4 K of a CO-treated, succinate-reduced cell sample, into which no O_2 had been stirred. The peak centred at 558 nm is due to *b*-type cytochromes and includes a small contribution from cytochrome *o* [9], whilst the band at 635 nm arises from CO–Fe^{2+} cytochrome *d*. Laser flash photolysis (fig.2B) caused a significant blue shift of the latter peak, attributed to photodissociation of the CO–*d* complex. The difference spectrum (fig.2C), obtained by subtraction of the two previous spectra, closely resembles the 'reduced minus CO-reduced' difference spectra in [16]*. A small peak at 445 nm is the weak Soret band of reduced cytochrome *d* [9]. Photolysis was essentially complete with one laser flash, as indicated by the lack of further change on repeated flashing. Exposure of the sample to the measuring beams for up to 70 min at 5 K caused no detectable photolysis.

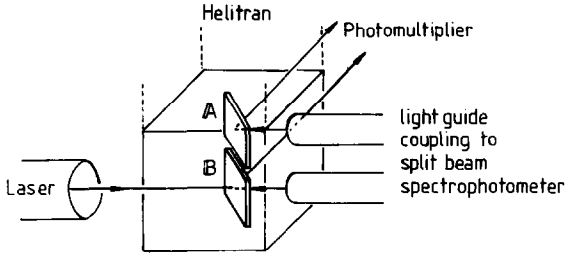

Fig.1. Optical configuration for reflectance spectrophotometry and flash photolysis at liquid He temperatures. The reference (A) and sample (B) cuvettes are inclined at 45° to the light paths of the laser, photomultiplier and measuring beams, and mounted in an evacuated chamber at the base of a Helitran temperature regulator.

* Photodissociation of carbon monoxyhaemoglobin, however, yields a form not identical with the unliganded ferrous form [5]

Photolysis at 4 K of a sample to which ~200 μM O_2 had been added before freezing resulted in a similar spectrum. Even at this temperature, difference spectra recorded at time intervals revealed a decrease in the bands attributed to reduced cytochrome *d*, indicative of ligand binding.

Two kinds of experiments were performed to determine whether the ligand binding was O_2 or CO:

(1) Photodissociated samples to which ligand was rebinding at either 5 K or 20 K (fig.3) were irradiated with light from a Xenon flash lamp. Reversion of the spectral changes to their original magnitudes suggested that the ligand binding in each case was CO, the much greater sensitivity of CO compounds, compared to O_2 compounds being well-established and assumed to hold in this case [17]. At 5 K, the recombination was apparently first-order, whilst at 20 K the kinetics were polyphasic. Complex recombination kinetics have also been observed in the reactions of CO with myoglobin [18] and the *Pseudomonas* cd_1-type cytochrome oxidase at room temperature [19] and very low temperatures [20].

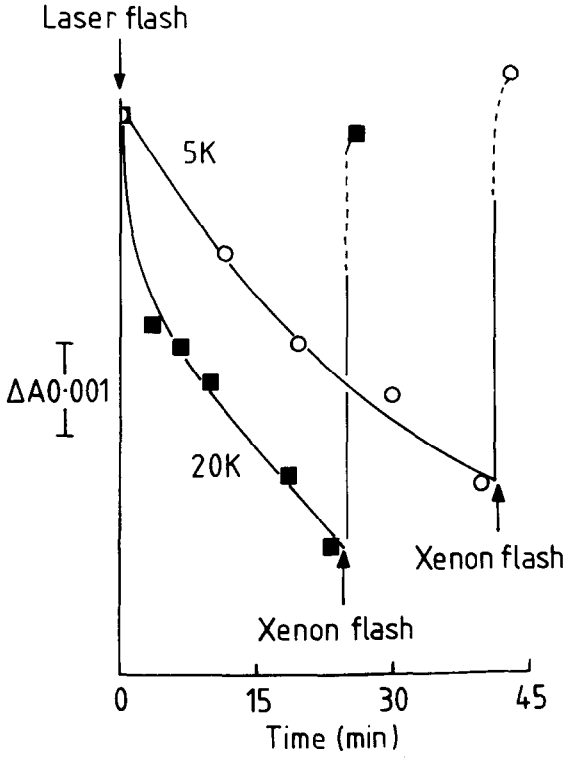

Fig.3. Kinetics of rebinding of CO to photodissociated carbon monoxycytochrome *d* at 5 K (—○—) and 20 K (—■—). Absorbance changes were measured at 624 minus 638 nm in difference spectra (photolysed minus CO-reduced). The reaction was initiated by one flash of the liquid dye laser and the samples further flashed with a Xenon arc lamp at the times shown. The sample (39 mg protein/ml), which also contained ~200 μM O_2, was warmed to 60 K between the experiments shown to allow complete CO recombination.

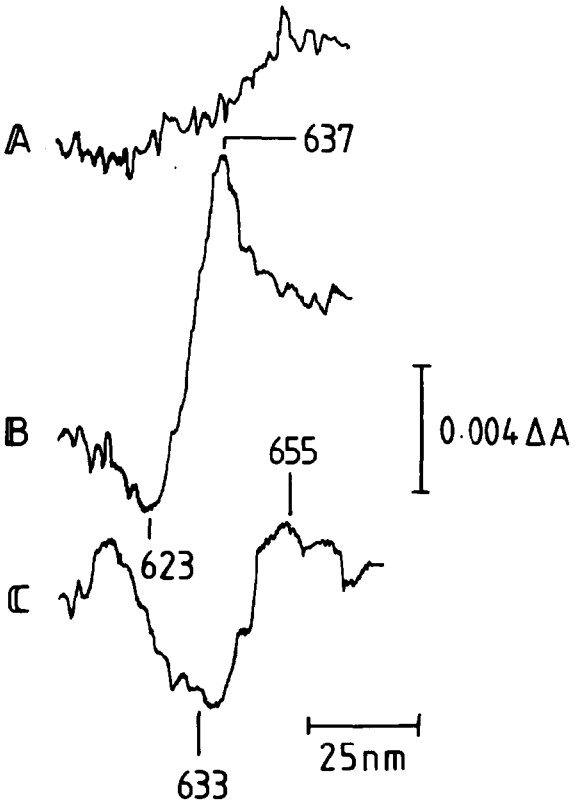

Fig.4. Identification of the ligand binding to cytochrome *d* after photolysis at 5 K and 166 K. Spectrum (A) is the difference between a sample, photolysed at 5 K, raised to 205 K and returned to 5 K, minus the preflash spectrum at 5 K, while (B) is similar but the reference spectrum is that of the sample immediately after photolysis. In (C) the preflash spectrum was recorded at 60 K, the sample taken to 166 K, and flashed there with the laser before returning to 60 K; the difference spectrum shown is post-flash minus pre-flash; protein was 42 mg/ml in (A) and (B) and 30 mg/ml in (C).

(2) Cytochrome *c* oxidase and myoglobin each form O_2 compounds that are spectrally similar to the respective CO compounds [21], so that the similarity of the prephotolysis and post-recombination spectra in the present experiments is not informative about the identity of the bound ligand at temperatures where further reaction of a presumptive O_2 compound would not be expected. As fig.4 (A,B) shows, however, raising the tem-

perature of a sample (which had been photolysed at 5 K) to 205 K (where ligand binding would be completed) and then recycling to 5 K did not reveal any spectral changes that could be attributed to electron transfer to oxygen. Similar experiments showed that following photolysis at temperatures upto 120 K, the recombining ligand was CO, not O_2.

When flash photolysis was performed at 166 K, however, we obtained evidence for a spectrally distinct form of cytochrome *d* that is unlike the reduced or ferricyanide-oxidized form (fig.4C). This is presumably an early, or perhaps the first, intermediate in the reaction of cytochrome *d* with O_2 [9,11]. Optical and EPR studies of this compound and its reactions will be presented elsewhere.

4. Discussion

Carbon monoxide has been used extensively as a probe for the structure and function of oxygen-binding haemoproteins since:

(i) Oxygen and carbon monoxide compete for the same active sites;
(ii) The rate of recombination of CO is generally slower than that of O_2;
(iii) The apparent quantum yield for photodissociation is much higher for CO than for O_2.

The CO complexes of many haemoproteins are rapidly formed from photodissociated samples at temperatures as low as 5–30 K, whereas the reduced cytochrome *c* oxidase of eukaryotic mitochondria recombines with photodissociated CO only above 150 K [4,5,21]. Here, we have demonstrated that this unusual behaviour is not related to the function of the enzyme, since photodissociation and recombination of CO at 5–120 K is exhibited by cytochrome *d*, a terminal oxidase in *E. coli* [13].

These results clearly distinguish cytochrome *d* from cytochrome oxidase aa_3 and indeed any other cytochrome oxidase so far described. It is tempting to suggest, as in [22], that an essential difference between cytochrome oxidase aa_3 and other haemoproteins is that the former contains Cu, which is capable of trapping CO photodissociated from the haem. Indeed, recent infrared interferometer experiments [23] have identified absorptions in cytochrome oxidase samples, attributable to Cu–CO, similar to those in haemocyanin from molluscs and crustaceans. There is no convincing evidence for the involvement of Cu in the respiratory chain of *E. coli* [8,24], consistent with the low temperature association of cytochrome *d* with CO reported here. An apparent anomaly is that the major alternative oxidase in *E. coli*, cytochrome *o*, is also widely believed to lack Cu and yet binds to CO only at $\gtrsim$170 K [12]. However, its O_2 binding is also sluggish at sub-zero temperatures and a comparison of cytochrome *o* with aa_3 with respect to the ratio of half-times for O_2 and CO binding at ~173 K reveals disparate behaviour. For the mitochondrial, Cu-containing enzyme, this ratio is ~60:1 (calculated from data in [21]), whereas for *E. coli* cytochrome *o* it is ~7:1 [12]. However, the present data do not rule out the possibility that protein residues, rather than proximal Cu, slow the rate of recombination of CO with cytochrome aa_3.

The fact that ligand exchange occurs after flash photolysis at 166 K is evidence for the close proximity of oxygen molecule(s) to the photolysed haem and which are ready to replace the CO molecule before it can return to its original position at the haem iron. Ligand exchange does not occur, however, at 4–20 K or on warming to 205 K a sample that had been photolysed at 5 K. Two classes of explanation are offered:

(1) Oxygen may not be in the haem pocket or cannot diffuse into the haem pocket due to its immobilization at 5–20 K;
(2) The CO ligand may not be displaced far from the iron by photolysis at these low temperatures but is displaced at 166 K, as appears to be the case for myoglobin (B. C., R. Fischetti, A. S., L. Powers, in preparation).

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